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14. ABSTRACT
The purpose of this study is to test the hypothesis that hypertrophic burn scars can be remodeled by fractional laser treatment and administration of stem cells. Finding the best ways to combine these approaches is a goal of this proposal. During the period of this report we have been completing an examination of the effect of fractional CO_2 and Erbium:YAG lasers on hypertrophic third degree burn scars made in Red Duroc pigs. Fractional lasers appeared to remodel the superficial dermis although deeper scarring could still be observed. Western blot analysis from tissue samples indicated that MMP9 and perhaps decorin play a role in this dermal remodeling. Fibroblasts grown from treated burn scars also supported these findings. Erbium:YAG laser seemed to be more effective than CO_2 laser in producing these changes. Early work examining the ability of fractional lasers to deliver stem cells has established that these lasers can get stem cells into hypertrophic scarred tissues. Delivering stem cells stem cells to burn scars also appeared to reduce the transformation of fibroblasts to myofibroblast. Although preliminary, allogeneic stem cells appeared to be superior in improving clinical scoring of hypertrophic third degree burn scars.

15. SUBJECT TERMS
fractional laser, stem cells, scarring, dermal remodeling

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INTRODUCTION: The purpose of this study is to develop an optimal delivery system for stem cells that can reduce burn scars. In this unique approach both the delivery system and delivered agents have been shown to have an effect in scar reduction. Combining these technologies could have a greater than additive effect on skin regeneration with normalization of function. New technologies that reduce burn scars would have a significant impact not only for wounded warrior, but also civilians who suffer from burn injuries. This proposal aims to evaluate the effectiveness of these novel delivery systems and cell-based therapies for third degree burns in a porcine model. We will test the hypothesis that hypertrophic burn scars can be remodeled by fractional laser treatment and administration of stem cells. Stem cells will be administered alone or incorporated into a chitosan fibrin matrix. Finding the best ways to combine these approaches is a goal of this proposal.

BODY: Our first task was to obtain final IACUC approval, which was amended for additional tissue sampling and minor changes. After approval was obtained, the first set of Red Duroc pigs were obtained and quarantined in accordance with University of Miami Miller School of Medicine IACUC guidelines. Prior to placing third degree wounds on the backs of pigs, we obtained our first source of adipose derived stem cells (ADSCs) and bone marrow derived mesenchymal stem cells (BM-MSCs). This would be a procedure that would continue throughout the study in order to obtain both autologous and allogeneic sources of stem cells. In this first preparation of cells we would validate the harvesting procedure ADSCs and BM-MSCs and confirm their phenotype at the University of Miami Miller School of Medicine and the USAISR. To obtain ADSCs, a sample of adipose tissue was surgically removed from the back (front shoulder) region under sterile conditions. The area chosen for adipose tissue harvesting was designed to be far from where the burn wounds would be placed and not interfere with healing. Bone marrow aspirate was taken from the long bones using a standard (sterile) clinical bone marrow aspiration kit. The adipose tissue and bone marrow aspirates were taken to the laboratory to isolate and expand ADSCs and BM-MSCs. Samples of these cells were sent to Dr Christy's laboratory. Analysis by flow cytometry in both laboratories confirmed the phenotype of both ADSCs and BM-MSCs and marked with similar antibodies as we have found in Yorkshire pigs. ADSCs and BM-MSCs were then labeled by lentiviral constructs that conferred nuclear fluorescence to cells once transduced. The transduction procedure was optimized and validated for Red Duroc ADSCs and BM-MSCs. Transduction was performed at a very early passage number to minimize overexpansion of the cells, which can alter their function. Transduced stem cells we evaluated and did not exhibit changes in growth or differentiation capacity.

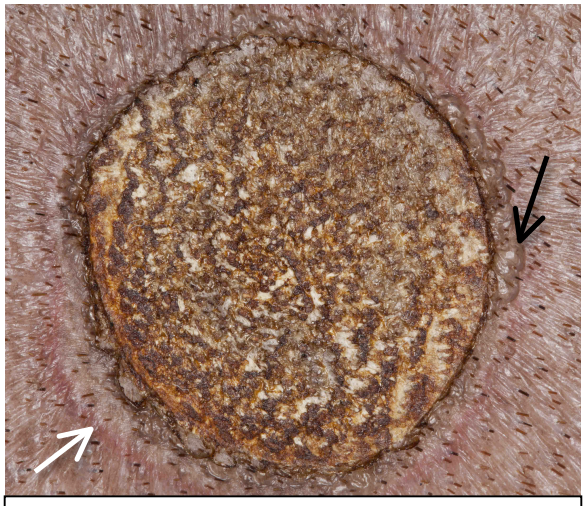

Figure 1: Third degree burn immediately after injury. There is loss of epidermis (char) and hair follicles. Blistering is noted around the wound (black arrow) and there is a surrounding region of erythema (white arrow).

Third degree burns were made in two Red Duroc pigs on the paravertebral and thoracic area by using a special branding iron (L & H Manufacturing Company Mandan, North Dakota 58554. Wounds were evaluated weekly for scar formation. Evaluation of these burns by Dr Carl Schulman (board certified burn surgeon at the University of Miami Miller School of Medicine) confirmed that these wounds were analogous to third degree burn wounds seen clinically in patients (Figure 1). Burn wounds would be allowed to develop into hypertrophic scars over 70 days prior to treatment with fractional laser. During this scar maturation phase, it was noted that the placement of wounds closer to hind or fore limbs of the animal resulted in a more linear arrangement of the resultant scar (Figure 2). While changes in clinical scar shape would not have significant impact on tissue sampling (which was always directed to harvest within scar material), wounds on subsequent animals were placed closer to the center of the animal and resulted in better overall similarity in clinical scar morphology.

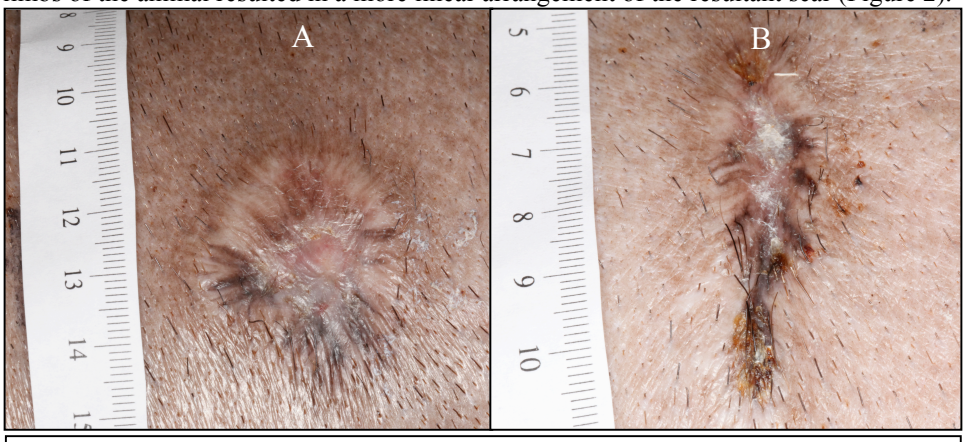

Figure 2: Burn scars 70 Days after injury. A: scar place more centrally in the animal has a more round shape. B: Scar placed closer to the front legs is more linear, likely due to skin tension in this region.

Histologic evaluation of wounds 70 days after burn injury is consistent with hypertrophic scarring. Burned areas are completely epithelialized and have minimal papillary dermis with underlying fibrosis indicative of a deep scarring process. Both Aldehyde Fuchsin and Elastin Van Gieson stains indicate the loss of elastic fibers, consistent with a scar. Twenty-seven third degree burn scars (70 days post burn injury) on each animal were divided into three groups. One pig would be treated with ablative fractional CO_2 laser with Group A receiving fractional CO_2 laser at a high setting, Group B receiving fractional CO_2 laser at a low setting and Group C as an untreated control. The CO_2 laser high setting was 30mJ at 3% density with the low setting 12.5mJ at 3% density. The other pig was treated with ablative fractional Erbium:YAG laser with Group A receiving fractional Erbium:YAG laser at a high setting, Group B receiving fractional Erbium:YAG

laser at a low setting and Group C as an untreated control. The Erbium:YAG laser high setting was 900μ at 22% density with the low setting 300μ at 22% density. The high and low settings for both lasers represent higher and lower settings that have been used clinically in patients.

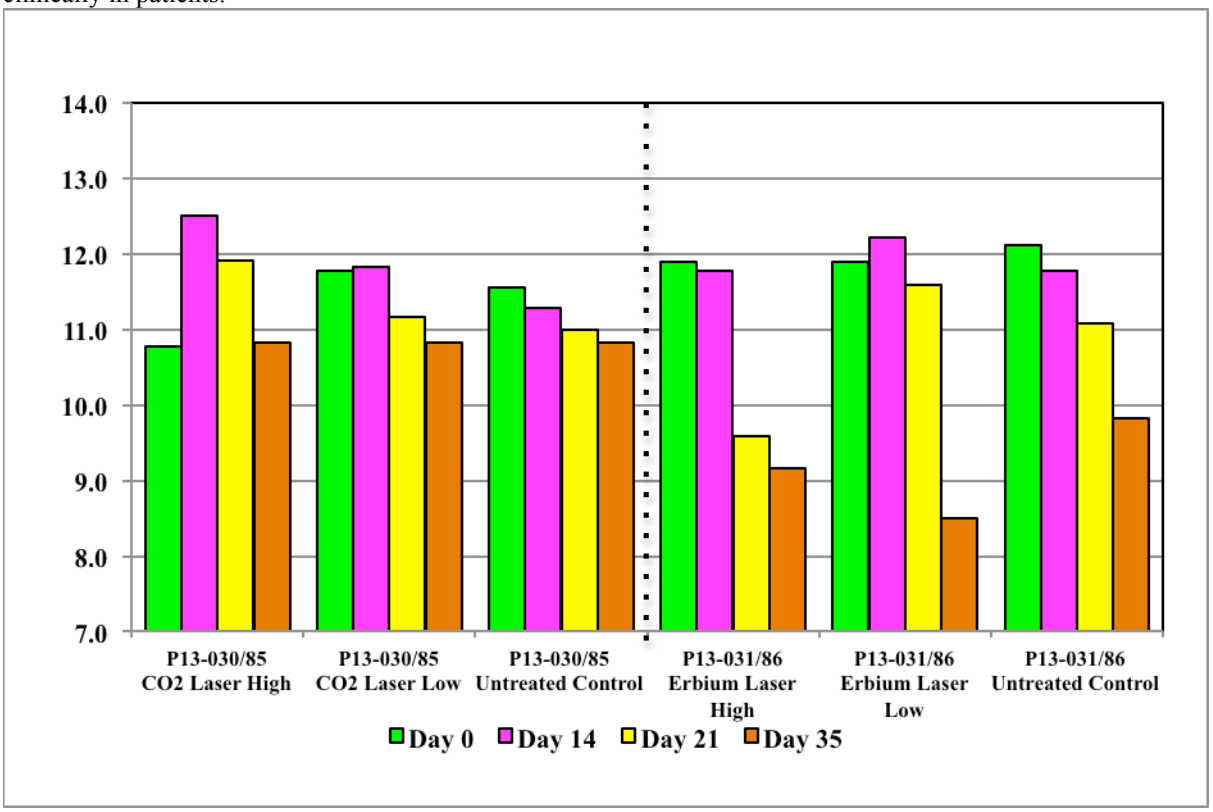

Figure 3. Burn scars evaluated by our modified scoring system. Lower scores indicate a better outcome with less scar like features. The first three sets of columns are scores derived from a pig receiving CO_2 laser at high setting, CO_2 laser at low setting and untreated control. The second three sets of columns are scores derived from a pig receiving Erbium:YAG laser at high setting, Erbium:YAG laser at low setting and untreated control. The Erbium:YAG laser appeared to have better outcome as compared to control at Day 35. The improvement with Erbium:YAG appeared to occur sooner (Day 21) at the higher setting however a greater improvement was noted at Day 35 with the low setting as compared to the high setting.

After burn injury and throughout the treatment phase we refined a clinical scoring system to grade scars. Grading was initially performed using both modified Vancouver and Manchester scar scales. Our goal was to develop two grading systems; one that would be performed on site clinically and the other that would allow blinded evaluators to assess photographs. Serial digital photographs were taken throughout the study and clinical evaluations made prior to each biopsy. The initial grading system took into account vascularity, pliability, color, contour, texture, and distortion. Wounds were evaluated by Dr Schulman who was blinded to all treatments. We found that some scoring criteria could be most affected by where the burn wound was placed and due to differences in skin color between individual Red Duroc pigs. As mentioned above, wounds placed closer to the front and hind limbs of the animals appeared more linear. This finding had a significant impact on the measurement of distortion. Variations in skin color between Red Duroc pigs had a significant impact on the evaluation of pigmentation. We therefore eliminated distortion and pigmentation from our scoring system. Our modified scoring system for on site clinical evaluation of burn scar treatment would then be based on grading vascularity, pliability, color, contour and texture. Photographic evaluation would consist of grading vascularity, color, contour and texture. Pliability was eliminated, as it could not be properly evaluated on photographs. By using these modified scoring systems, evaluation of burn scars became more uniform prior to treatment and throughout the study. Burn scars treated with Erbium:YAG lasers appeared to have improved scores over control treated wounds 35 days after treatment with both the higher and lower settings. The higher setting showed improvement earlier (Day 21) than the lower setting however the overall improvement appeared better at Day 35 with the lower setting (Figure 3). Some improvement was noted from Day 0 to Day 35 with CO_2 laser at the lower setting; however, a similar improvement from Day 0 to Day 35 was also noted in the control samples. Interestingly, there was improvement in the controls from Day 0 to Day 35 in both animals (one treated with Erbium:YAG and the other with CO_2 laser), which could indicate a possible systemic effect mediated by laser treatments.

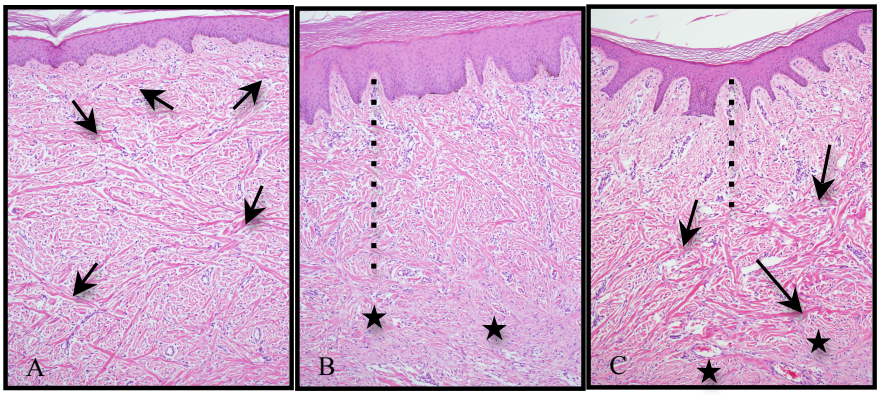

Figure 4: Burn wounds 35 Days after treatment. A: Control burn wound. B: Fractional CO_2 treated burn wound. C: Erbium-YAG treated burn wound. The arrows highlight thickened collagen bundles indicative of hypertrophic scar or keloid formation. The dotted lines indicate the areas of dermal remodeling. The stars highlight the edge of the deep dermal scar in the laser treated wounds. The control wound (A) has thickened collagen bundles extending throughout the superficial, mid and deep dermis. There is minimal evidence of superficial dermal remodeling. The epidermis is thicker in both fractional CO_2 (B) and factional Erbium-YAG (C) treated wounds. The fractional CO_2 treated wound had less dense collagen bundles in the superficial and mid dermis indicative of scar remodeling. The Erbium-YAG treated burn wound had less dense collagen bundles in the superficial dermis but there are thickened bundles noted in the mid reticular dermis (examples highlighted by arrows).

Biopsy samples were taken from burn scars 14, 21 and 35 days after treatment. Deep dermal scar formation was noted in all treated samples and controls (Figure 4). In control wounds the epidermis overlying scars tended to be thinner than that seen in the laser treated samples. There was a tendency to observe thickened (keloidal) collagen bundles throughout the superficial, mid and deep dermis in control burn scars. Samples treated with both fractional lasers tended to exhibit a superficial area of dermal remodeling. These features were noted most prominently at Day 35 (Figure 4 and Figure 5). The areas of dermal remodeling tended to be greater in the CO_2 laser treated burn scars than those treated with Erbium:YAG. The Erbium:YAG treated burn scars however had finer collagen bundles in a more random arrangement in the areas of dermal remodeling. This could explain why the Erbium:YAG treated scars tended to score better clinically (Figure 3). Aesthetic scoring is likely to be most influenced by changes in the superficial changes in the scar. A thicker epidermis and more soft or pliable superficial dermis would be expected to be consistent with a better cosmetic appearance and feel. This could be a limitation based on the penetration depth capable by these laser devices. A method to address the deeper scarring process is needed, as these deep scars are responsible for much of the morbidity associated with deep burns. Optimizing the penetration of these fractional lasers without creating more thermal damage might be one approach to this problem but this may necessitate the development of newer devices. .

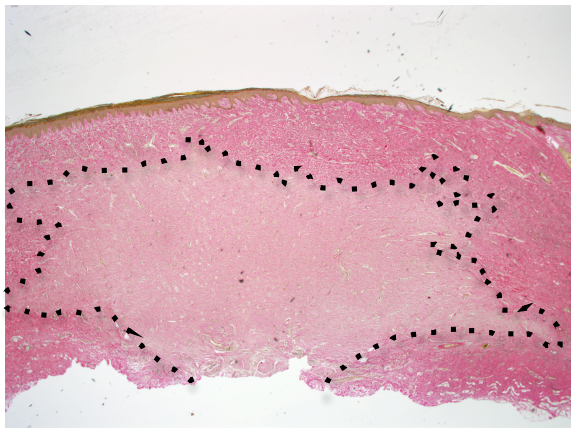

Figure 5: Elastin Van Gieson stained section of a third degree burn wound treated with CO_2 laser, Day 35. The dotted line highlights the burn scar in the dermis. Despite overlying dermal remodeling, as significant deep dermal burn scar remains.

Biopsy samples were also prepared for analysis of nucleic acid and protein expression. We examined methods for simultaneous extraction of nucleic acids and protein from scar tissue samples. Commercial kits for isolation of RNA, DNA and protein were examined and compared to protein only extraction methods we have traditionally used in our laboratory. All comparisons were performed on Duroc pig samples. While nucleic acid and protein kits appear to perform well for isolation of RNA and DNA, protein quantity was below what we are able obtained by protein (only) isolation methods used in our laboratory. We therefore elected to process snap frozen tissue samples for protein extraction. Nucleic acid analysis would be performed on fibroblasts grown isolated in tissue culture from fresh tissue samples of treated burn scars.

Protein expression analysis from snap frozen tissue samples was performed using porcine specific antibodies. Existing antibodies specific for detection of many porcine antigens are however limited. While there are several commercial sources, we have found that many available antibodies are not particularly specific and/or failed to produce optimal results in Western blot analysis, direct immunofluorescence (on frozen sections) or immunohistochemistry (frozen and/or paraffin embedded sections). We have therefore had to screen several antibodies to evaluate their performance and specificity. Commercial sources for reliable antibodies directed against transforming growth factor beta 1 (TGF-βI), matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), smooth muscle actin (SMA), and decorin have been identified. We are continuing to screen additional antibodies. Nucleic acid analysis would rely on development of porcine specific primers. We have developed and verified porcine specific primers for Collagen I, Elastin 1 & 2, MMP2 & 9, TIMP1 & 2, TGF-βI and SMA. Primers for Collagen IV and TGF-βIII are being developed.

Samples were also prepared by cryosectioning for direct immunofluorescence analysis. Matrix metalloproteinase 9 (MMP9) was noted to be greater in the area of (superficial) dermal remodeling in the laser treated burn scars (FIGURE 6). In burns treated with Erbium:YAG lasers, increase in MMP9 appeared to be greatest 21 days after treatment and continued to be elevated at day 35. Burn wounds treated with Erbium:YAG at the lower setting had a higher level of expression than those treated with the higher setting. Burn wounds treated with CO_2 laser also appeared to have elevated MMP9 at Day 35 but to a lesser extent than seen in Erbium:YAG laser treated wounds (Figure 7). MMP9 in these samples may be important in remodeling the dermis in this area by breaking down scar material. These findings are also consistent with the observation that Erbium:YAG treated burn scars scored better clinically. The

effect of MMP9 might also be see histologically by the presence of fewer thickened collagen fibers in the area of remodeling as compared to somewhat less remodeled areas in CO_2 treated burns or controls which had little if any remodeling.

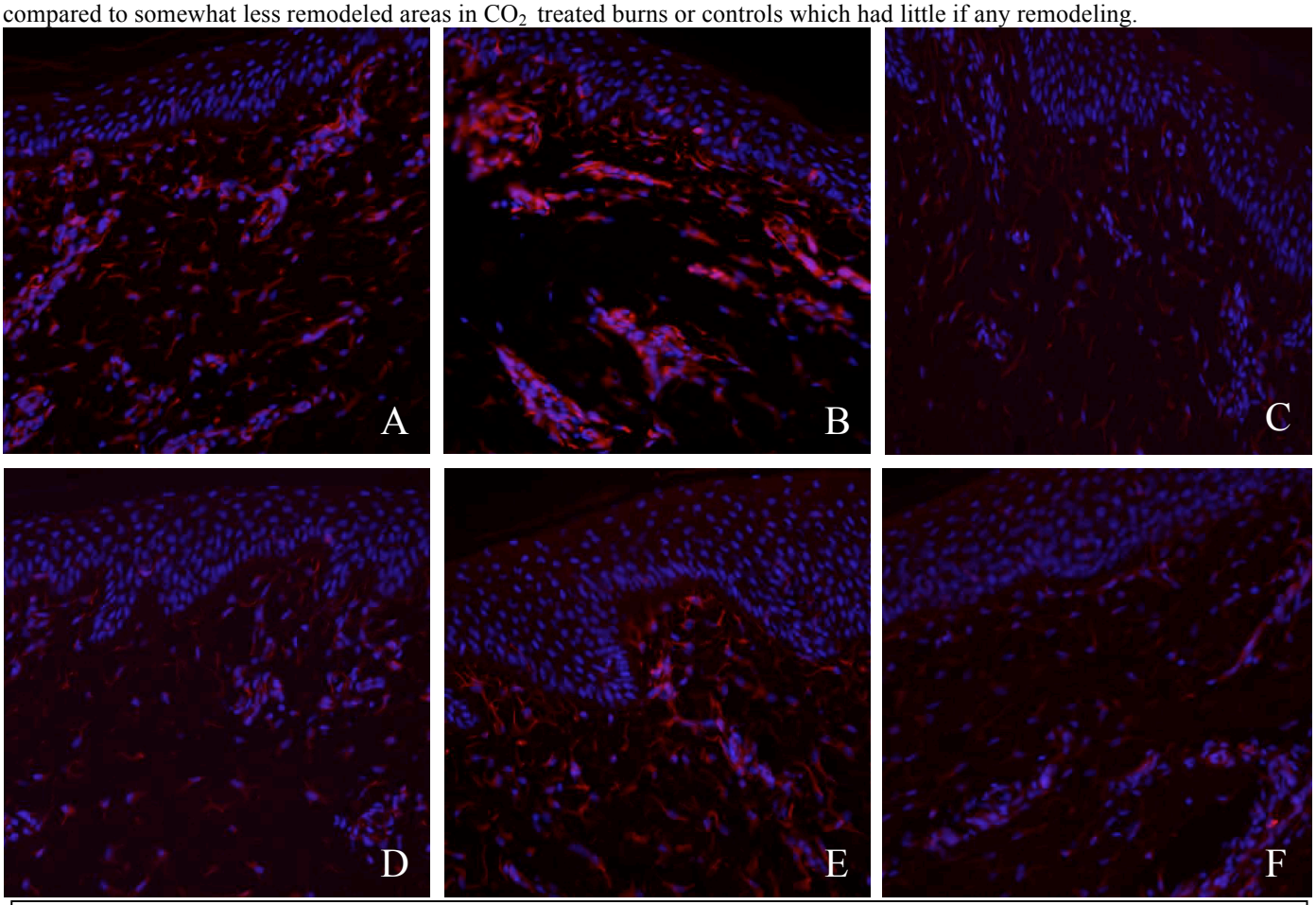

Figure 6: Direct immunofluorescence images of biopsies taken from an animal treated with Erbium:YAG laser at high setting (A and D), Erbium:YAG laser at low setting (B and E) and untreated control (C and F). Samples A, B and C were taken 21 days after treatment. Samples D, E and F were taken 35 days after treatment. All sections have been stained with DAPI (blue) and MMP9 (red). MMP9 expression appears to be greater in the region of dermal remodeling in the laser treated burn scars at Day 21 for both high and low settings. Expression of MMP9 continues to be higher in the remodeling region at Day 35 however appears greater for the burn wounds treated with the lower setting.

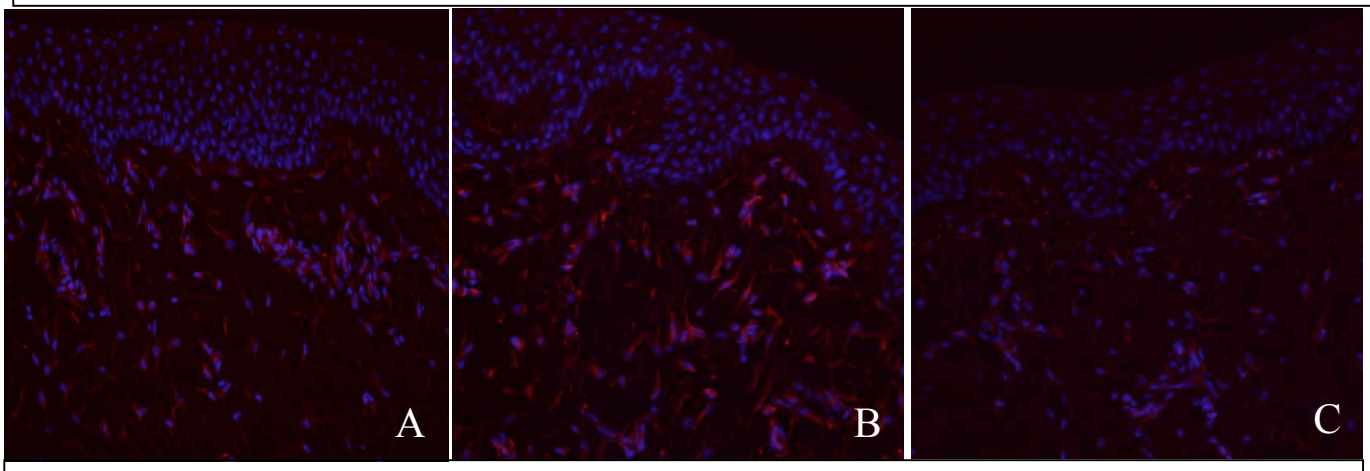

Figure 7: Direct immunofluorescence images of biopsies taken from an animal treated with CO_2 laser at high setting (A), CO_2 laser at low setting (B) and untreated control (C). Samples A, B and C were 35 days after treatment. All sections have been stained with DAPI (blue) and MMP9 (red). MMP9 expression appears to be greater in the region of dermal remodeling in the laser treated burn scars. Expression of MMP9 appears greater for the burn wounds treated with the lower setting.

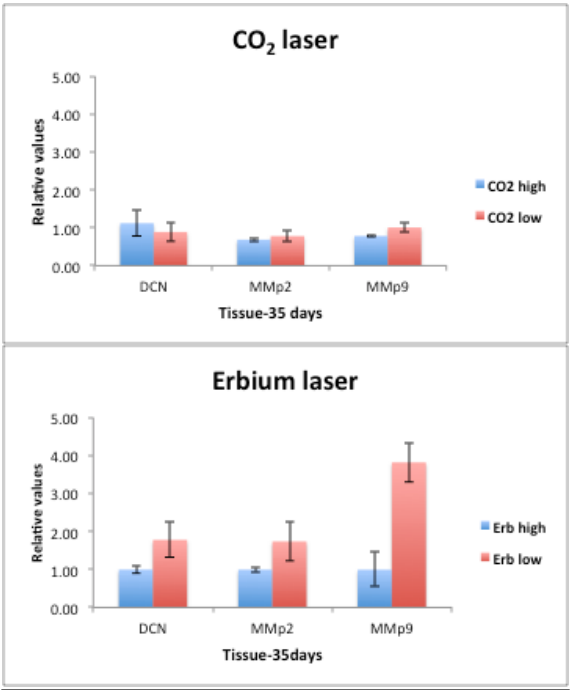

Figure 8: Western blot densitometry analysis of tissue total protein extracts at Day 35 for CO2 laser treated burn scars (above graph) and Erbium:YAG treated burn scars (graph below). All time points have an n=3 and are plotted relative to the untreated control burn scars (a relative value of 1 is equivalent to control).

Additional samples from each time point were snap frozen and processed for Western blot analysis. These samples were punch biopsies placed within clinically fibrotic areas of the burn scar. At Day 35 there appeared to be mild elevation of decorin above that seen in control burn scars in some (but not all) scars treated with CO_2 laser at the higher setting. There was a more consistently elevated level of decorin expression at Day 35 in burn wounds treated with Erbium:YAG laser at the lower setting. A mild elevation in MMP2 over controls was noted in burn scars treated with Erbium:YAG laser at the lower setting at Day 35. The most significant difference was however noticed in a larger increase in MMP9 (over controls) in burn wounds treated with Erbium:YAG laser at the lower setting (Figure 8). These findings also support the possible role of MMP9 in dermal remodeling seen with fractional laser treatment, particularly for Erbium:YAG laser.

Additional biopsies were taken at the same time points to isolate and grow tissue fibroblast from the treated and control burn scars. Tissue samples (3 per time point) were decontaminated using several washes developed in our laboratory and pooled to ensure there would be enough material to provide reliable growth of cells. We had found in previous work that single punch biopsy specimens from porcine skin did not provide reliable fibroblast growth. Combining several punch biopsies samples however greatly increased our ability to obtain enough fibroblasts in tissue culture for future analysis. We were successful here in growing fibroblasts from all time points in both laser treated animals. Protein and nucleic acid extraction was performed early on cells from each time point at cell passage 1 (P1). At P0 we noticed some unique features in cells derived from laser treated burns (Figure 9). Fibroblasts derived from laser treated burn scars grew faster than those derived from untreated control scars with fibroblasts derived from CO_2 laser treated wounds growing faster than those from Erbium:YAG treated wounds. Fibroblasts from laser treated wounds also exhibited two morphologies with one group have a more traditional fibroblast appearance (spindle and stellate cells) and the other group growing in a more linear or network like arrangement. Fibroblasts derived from control burn scars did not exhibit this linear/network morphology. As the cultures became confluent and progressed to P1 however the linear/network morphology could no longer be easily identified in the laser treated fibroblasts. This may have been due to these cells being crowded out in flasks as they became more confluent. In passing cultures containing the linear/network morphology we could not re-establish this growth pattern.

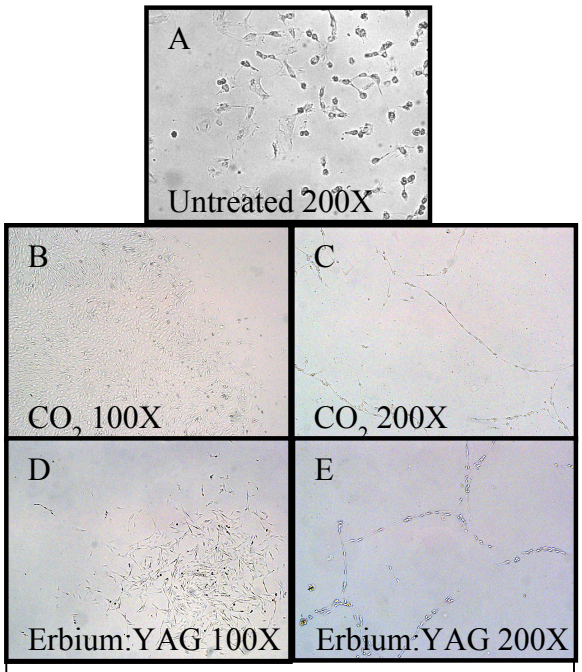

Figure 9: P1 fibroblast cultures derived from a control wound (A), fractional CO_2 laser treated wound (B & C) and Erbium:YAG treated wound (D & E). Colonies of more traditional appearing fibroblast-like cells were noted in both laser treated groups (B&D) however colonies derived from fractional CO_2 laser treated wounds appeared to grow faster than those derived from factional Erbium:YAG laser treated wounds. In examples C & E, a population of cells in a linear, somewhat network, arrangement were noted in both fractional CO_2 and factional Erbium:YAG laser treated wounds. This was not observed in control wounds (A).

At P1 the cells were harvested and processed for nucleic acid and protein extraction. Protein expression was examined by Western blot analysis. An elevation in decorin (relative to controls) was noted at Day 14 in cell derived from burn scars treated with Erbium:YAG laser at both the low and high settings (Figure 10). This increased expression in decorin was only seen at Day 14 and fell to levels below controls at Days 21 and 35. An earlier rise in decorin expression could represent a more immediate post treatment response to remodel scars that dissipates after two weeks (figure 10). This could also explain why reports treating burn scars with fractional lasers required multiple treatments, as the benefits from each treatment could be rapid but short lived. MMP9 protein expression in cells derived from Erbium:YAG treated burn wounds indicated a significant increase in MMP9 at Day 21 (Figure 11). This correlated well histologic findings. While cellular expression fell by day 35, tissue levels of MMP9 remained elevated at Day 35. At Day 35, levels of TGFβI was lower (relative to controls) in cells derived from burn scars treated with CO_2 and Erbium:YAG at both high and low settings. We are currently performing real time PCR analysis for RNA expression on cells derived from burn scars. Preliminary results for CO_2 laser treated burn scars at Day 35 indicate decreased expression of TGFβI (both high and low setting) and increase expression of MMP9, αSMA and MMP2. We are continuing to analyze these samples.

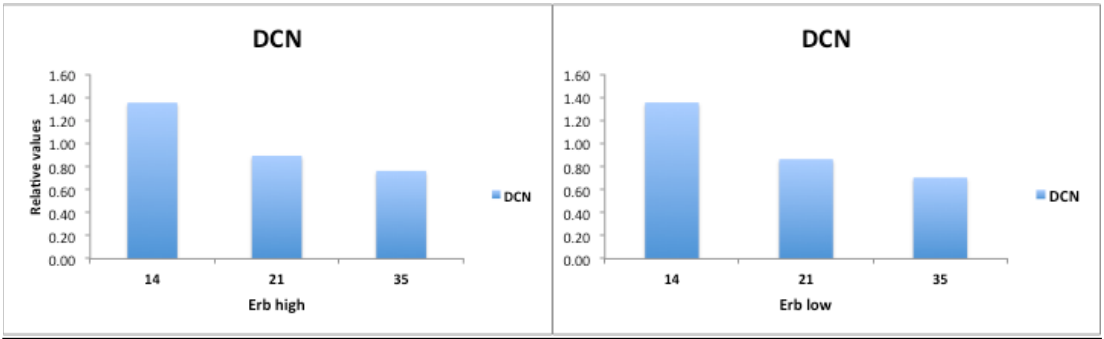

Figure 10: Western blot densitometry analysis of burn scar fibroblasts derived from Erbium:YAG treated burn scars relative to untreated control burn scars on Days 14, 21 and 35 post treatment. Graph on left is the analysis of decorin expression in burn scars treated with Erbium:YAG laser at the high setting. Graph on right is the analysis of decorin expression in burn scars treated with Erbium:YAG laser at the low setting.

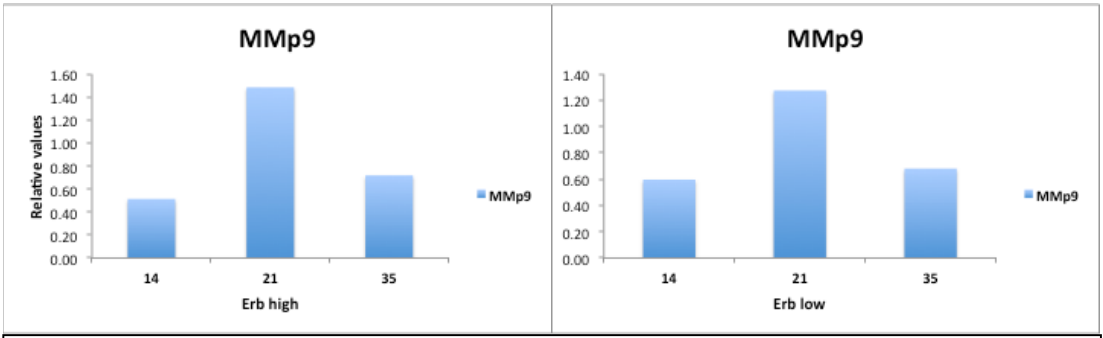

Figure 11: Western blot densitometry analysis of burn scar fibroblasts derived from Erbium:YAG treated burn scars relative to untreated control burn scars on Days 14, 21 and 35 post treatment. Graph on left is the analysis of MMP9 expression in burn scars treated with Erbium:YAG laser at the high setting. Graph on right is the analysis of MMP9expression in burn scars treated with Erbium:YAG laser at the low setting.

A second set of three animals was also prepared for treatment with fractional laser plus the direct application of stem cells to begin work on Specific Aim 2. Prior to creating burn injury, each animal had undergone adipose tissue harvest and bone marrow aspiration. ADSCs and BM-MSCs were isolated from these samples and characterized by flow cytometry to confirm their phenotype. These cells were then labeled using lentiviral constructs (as above) conferring YFP nuclear fluorescence to transduced cells. Transduced stem cells were confirmed to not demonstrate changes in growth or differentiation capacity. Isolating stem cells from these animals was necessary as an autologous source of stem cells was required for this experiment. Third degree burn scars were created similar to those described above for the first two animals treated. In this case however we made an effort to better centralized burn injuries and avoid areas closer to the front and hind legs. While there was some variability of scar shape, it appeared less than we had observed in the first two animals. Dr Carl Schulman again assessed the developing and treated burn scars in a blinded fashion. We confirmed that the burn injuries were in line with human 3rd degree burn injuries and that the scar formation was also consistent with human hypertrophic burn scarring. Scars were allowed to develop for 69 days after burn injury. Each animal then had their burn scars treated with fractional CO_2 laser plus stem cells, fractional Erbium:YAG laser plus stem cells and or left untreated (control). Each animal differed in the stem cells administered. One animal received autologous YFP-labeled BM-MSCs, one received allogeneic YFP-labeled BM-MSCs and the other received autologous YFP-labeled ADSCs. All cells were administered at P2 in order to maintain maximal stem cell phenotype. Samples of transduced cells were also sent to Dr Christy's laboratory for analysis and use in developing PEGylated matrices containing stem cells.

In the design of this experiment an effort was made to maximize the laser conduits for cell delivery. We also had no direct evidence that either CO_2 or Erbium:YAG laser would be superior for cell delivery to scarred tissue. We therefore elected to use both lasers on each animal at the higher setting, as this would produce larger channels that might allow more cells to be delivered deeper into tissues. The CO_2 laser setting was 30mJ at 3% density and the Erbium:YAG laser setting was 900µ at 22% density (both as performed in the high setting of the laser only experiments).

Photographic archiving of burn wounds as they progressed through the treatment process was performed. As in the previous study, Dr Carl Schulman performed blinded scoring of the treated and control wounds in each animal throughout the treatment period. We utilized the scoring system developed in the previous experiments. As in the previous experiment, control samples tended to have improved scar scoring at Day 35 in all animals, indicative of a possible systemic effect of these treatments (Figure 12). This potential systemic effect appeared to have been greatest when autologous cells were delivered. Improvements in clinical scar scoring over controls at Day 35 were noted for autologous BM-MSCs with Erbium:YAG laser, allogeneic BM-MSCs with CO_2 laser and allogeneic BM-MSC's with Erbium:YAG laser. Scar score improvement was noted earlier at Day 21 for autologous BM-MSCs with Erbium:YAG laser. We were however surprised to see that the best overall improvement in scar score (over controls) was noted with allogeneic cells, in this case BM-MSCs. If this hold true in further experiments, it could be particular significance as it may favor the use of a more readily available "off the shelf" supply of healthy donor cells to wounded warriors. This could avoid the need to utilize autologous stem cell resources that might be adversely affected by comorbidities.

As in our previous experiments, harvesting of treated and control burn wounds were performed at Days 14, 21 and 35. Biopsy samples were prepared for formalin fixation and paraffin embedding while other samples were prepared for frozen sections. Samples prepared for cryosectioning had to be specially processed using a paraformaldehyde/sucrose fixation procedure developed in Dr Christy's laboratory. This fixation procedure helps to preserve tissue integrity for better sectioning and without greatly altering the

nuclear fluorescence that would be present in the delivered transduced stem cells. Initial examination of sections prepared by this method has confirmed that delivered transduced cells can be detected without staining of the sections (Figure 13).

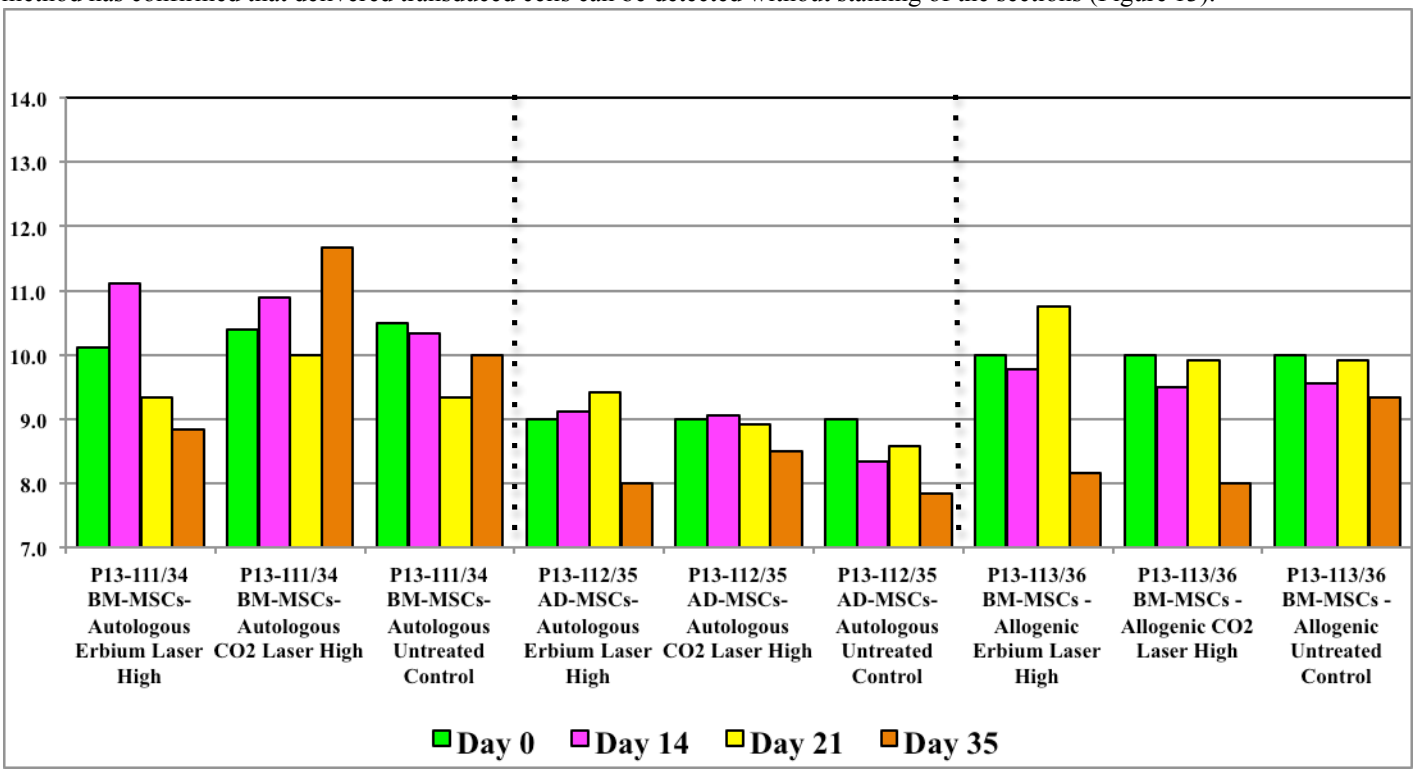

Figure 12: Burn scars evaluated by our modified scoring system. Lower scores indicate a better outcome with less scar like features. The first three sets of columns are scores derived from a pig receiving autologous BM-MSC delivered by Erbium:YAG laser, CO_2 laser and untreated control. The second three sets of are scores derived from a pig receiving autologous ADSCs delivered by Erbium:YAG laser, CO_2 laser and untreated control. The last three sets of columns are scores derived from a pig receiving allogeneic BM-MSC delivered by Erbium:YAG laser, CO_2 laser and untreated control. The allogeneic BM-MSCs appeared to have better outcome as compared to control at Day 35.

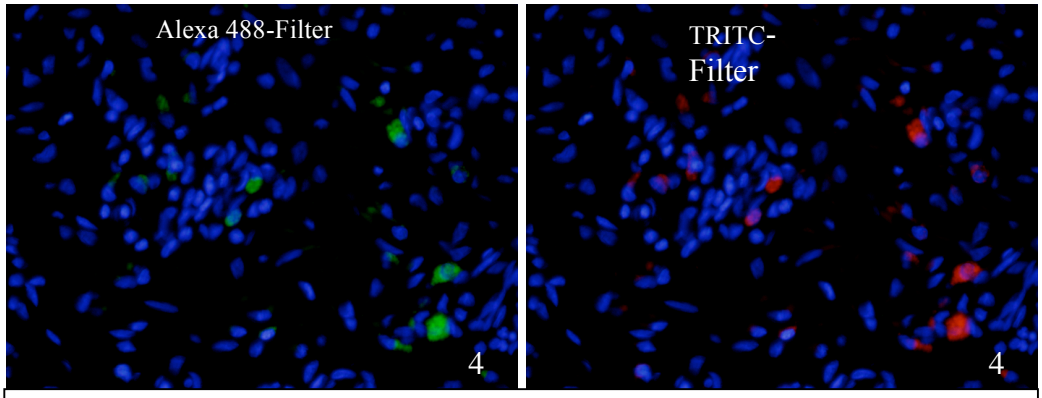

Figure 13. Burn scar14 Days after treatment with CO_2 laser and autologous BM-MSC. The section is stained with DAPI (blue) and also shows the native fluorescence of the labeled BM-MSCs delivered by laser using two different filters.

We have also begun to perform immunohistochemistry analysis of frozen sections prepared from these animals. In the previous experiment, Dr Christy's laboratory had performed direct immunofluorescence on frozen sections. While these studies led to several important preliminary findings, direct immunofluorescence does not allow for full evaluation of the overall histological architecture within a section. Immunohistochemistry (by using chromophores and visible light) provides much more histologic detail that could be important in evaluating dermal regeneration. Initial results have been favorable in evaluating overall histology, allowing for better identification of areas with active scarring and remodeling. In examining some sections 14 Days after injury, we have able to identify the laser channels (Figure 14). TGFβI expression within the laser channels treated with stem cells was increased and likely represents a healing response within the channels. In (unrelated) previous experiments, we have observed a similar healing response in stem cell treated laser conduits made in non-scared skin. Preliminary immunohistochemistry analysis has also revealed a reduced αSMA expression in burn scars treated with laser and autologous BM-MSCs (Figure 15). This indicates a reduced transformation of fibroblasts to myofibroblasts in the treated burn scars. Myofibroblast transformation has been closely associated hypertrophic scar formation and contraction. We are continuing to stain tissue sections from these animals and are optimizing the staining conditions for each antibody.

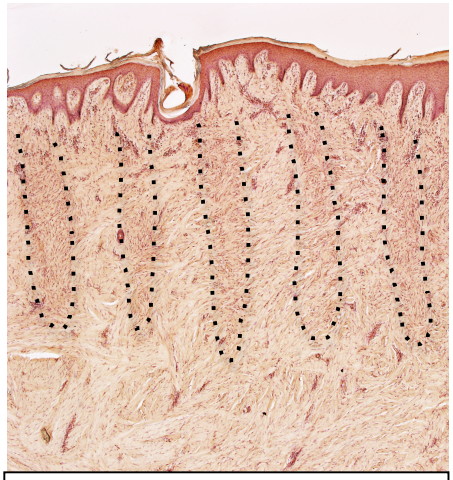

Figure 14. Burn scar14 Days after treatment with CO_2 laser and autologous BM-MSC. The section is stained for TGFβI. Dotted lines indicate laser channels within the treated scar where stem cells were delivered. There is increased staining within the channels indicative of activation .

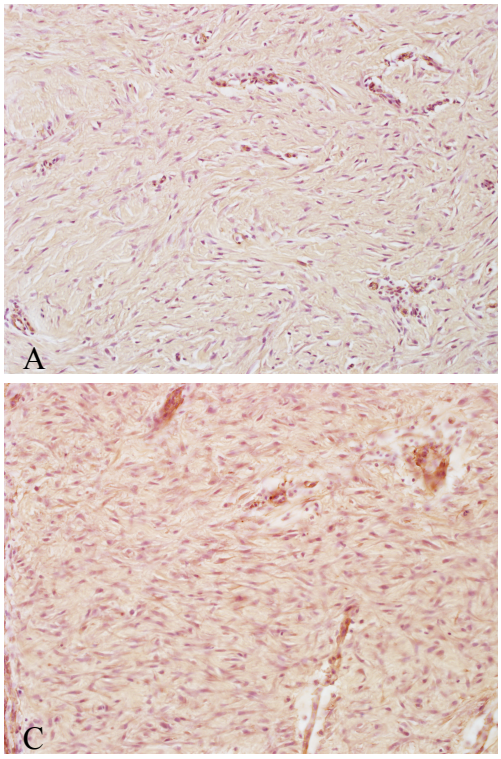

Figure 15. Burn scars 35 Days after treatment stained for αSMA. A: CO_2 laser and autologous BM-MSC treated burn scar. B: Erbium:YAG and autologous BM-MSC treated burn scar. C: untreated control burn scar. αSMA positive cells are indicative of fibroblast transformation to myofibroblasts. There are many more αSMA positive cells within the control (C) than in either laser and stem cell treated samples (A&B).

Frozen tissue samples for Western blot analysis have been snap frozen and stored for protein extraction. We will begin processing these materials.

Burn scar fibroblasts have been grown from all time points. As in the previous experiment, P0 cultures of cells derived from laser treated burns had the same unique features. Fibroblasts from the laser and stem cell treated burn scars grew faster than controls. They also had the same two morphologies as was observed in the previous experiment, which was not observed in controls. As the cultures progressed to P1, the two populations could not be identified as was observed before. The type of stem cells administered (autologous, allogeneic, adipose or bone marrow) did not seem to alter the appearance of the cultures. It again appeared to be predominately a feature associated with laser treatment.

KEY RESEARCH ACCOMPLISHMENTS:

- Red Duroc pigs obtained and appropriately quarantined
- Successful harvesting BM-MSCs and ADSCs
- Efficient transduction of BM-MSCs and ADSCs without functional loss
- Reproducible third degree burn wounds made in Red Duroc pigs.
- Centralizing burn wounds in animals yielded more uniform scar shapes
- Clinical scoring system established for third degree wounds in Red Duroc pigs
- Scoring system for photographic analysis of burn wounds established
- Controls in laser treated animals showed improvement, possible systemic effect of lasers
- Erbium:YAG laser produced better clinical scores in treated burn scars
- Erbium:YAG laser at lower setting had a greater effect than high setting
- Superficial dermal remodeling noted in the laser treated burn scars
- CO_2 laser appeared to have larger depth of remodeling but contained more fibrosis than Erbium:YAG treated burn scar
- Dermal remodeling with Erbium:YAG laser contained finer collagen bundles in a random arrangement.
- Deep scarring was noted in all samples, control and (both) laser treated
- MMP9 expression appeared greater in areas of dermal remodeling
- MMP9 expression in the remodeled areas was greatest at Day 21 for the Erbium:YAG laser and continued to Day 35
- MMP9 expression in the remodeled areas was higher at the lower settings for CO_2 laser at Day 35 but was not as highly expressed as that seen for Erbium:YAG treated burn scars
- MMP9 and MMP2 protein expression by whole tissue Western blot analysis was greater than controls at Day 35 in the Erbium:YAG treated burn scars at the low setting
- Decorin appeared increased at Day 35 in the CO_2 laser high setting and Erbium:YAG low setting treated burn scars
- Fibroblasts derived from burn scars treated with fractional laser (CO_2 or Erbium:YAG) grew faster than those derived from untreated control burn scars

- Two colony morphologies were noted in early passage tissue culture in fibroblasts derived from fractional laser (CO_2 or Erbium:YAG) treated burn scars but not in those derived from controls
- Decorin was elevated in cells fibroblasts derived from Erbium:YAG treated burn scars (at both high and low settings)
- MMP9 was elevated in cells fibroblasts derived from Erbium:YAG treated burn scars (at both high and low settings)
- Three Red Duroc pigs obtained and appropriately quarantined to begin laser delivery of stem cells experiment
- Harvesting of BM-MSCs and ADSCs performed to obtain autologous stem cells for this experiment
- 3rd degree burn wounds created more centrally in animal with less variation in burn scar shape
- Each animal treated with CO_2 and Erbium:YAG at high setting to maximize depth of cell delivery
- Laser treated burn scars on each animal were treated with different stem cells (one receiving autologous BM-MSCs, another receiving allogeneic BM-MSCs and the last receiving autologous ADSCs)
- At Day 35 all control burn scars scored better clinically, suggestive of a systemic effect
- Improvements in clinical scar scoring over controls at Day 35 were noted for autologous BM-MSCs with Erbium:YAG laser, allogeneic BM-MSCs with CO_2 laser and allogeneic BM-MSC's with Erbium:YAG laser
- Overall, burn scars treated with autologous stem cells (BM-MSCs delivery by CO_2 or Erbium:YAG) scored better than burn scars treated with autologous stem cells
- Immunofluorescence studies confirmed that stem cells were delivered to tissues using fractional laser
- Preliminary results of burn scars treated with fractional laser and stem cells indicate activation of a healing response within the channels as evidenced by TGFβI staining
- There is reduced transformation of fibroblasts to myofibroblasts (noted at Day 35) in burn scars treated with autologous BM-MSCs and fractional laser (CO_2 or Erbium:YAG) as evidenced by αSMA staining

REPORTABLE OUTCOMES:

2012- Military Health System Research Symposium, Fort Lauderdale, FL, Poster: Development of a Third-Degree Burn Wound Model to Evaluate Scarring and the Potential for Lasers as a Treatment Modality. Davis SC[1], Waibel JS[1], Christy RJ[2], Schulman CI[3], Gil J[1], Ford, BM, Natesan S[2], Valdes J[1], Treu R[1], Solis M[1], Salgado M[1], Rodriguez-Menocal L[1], Shabbir A,[1] Badiavas EV[1]University of Miami, Miller School of Medicine Department of Dermatology and Cutaneous Surgery, Miami, FL, † US Army Institute of Surgical Research, Fort Sam Houston, TX

CONCLUSION: During the first year we have evaluated the effect of fractional CO_2 and Erbium:YAG lasers on well formed third degree burn scars in Red Duroc pigs. A reliable scoring system was developed to evaluate scarring in this model. With this scoring system we have documented clinical improvement in treated burn wounds. We have examined how this clinical improvement is reflected in histologic, cellular and molecular changes. Fractional lasers appear to have an effect on superficial dermal remodeling in these scars. From a histologic perspective, deep scarring is mostly unaffected. There were some molecular changes in whole tissue protein expression that do however suggest there may be a deeper effect. Improvement in all untreated controls (which were placed in the same animal) suggests a systemic effect. Molecular changes noted in the areas of dermal remodeling indicated that MMP9 and decorin could play significant roles in tissue regeneration induced by fractional laser treatment. Erbium:YAG laser appeared to be better at inducing these changes than CO_2 laser. Fibroblasts isolated from treated burn scars also supported these findings. Fibroblasts derived from laser treated burn scars appeared to be more stimulated in culture, growing faster and expressing distinct colony morphologies. We have next begun an analysis of stem cells delivered to burn scars by CO_2 and Erbium:YAG fractional lasers. We have demonstrated that fractional lasers can deliver stem cells to hypertrophic scars. Clinical scoring of burn scars treated with fractional lasers and stem cells was performed. Although preliminary, burn scars treated with allogeneic cells and scars that had stem cells delivered by Erbium:YAG laser appeared to score better. Soon (14 days) after treatment, laser channels that had stem cells administered to them appeared stimulated (by evidence of TGFβI expression) to a greater extent than the surrounding scar. Burn scars treated with bone marrow derived stem cells (delivered by CO_2 or Erbium:YAG laser) also had less expression of αSMA indicative of a reduced transformation of fibroblasts to myofibroblasts. The transformation of fibroblasts to myofibroblasts has been cited as a major factor in hypertrophic scarring and contraction.
These findings have helped to illustrate the ability and mechanisms of fractional lasers in altering burn scars. By continuing our examination of combining stem cell delivery with fractional lasers we hope to maximize the benefit of these two technologies

REFERENCES:
None listed

APPENDICES:
None

SUPPORTING DATA: Figures have been incorporated into the body text.